

***In vitro* insulintropic actions of various extracts of *Moringa oleifera* leaves**

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ABSTRACT

This study investigated the *in vitro* insulin-releasing and cytotoxic effects of various extracts of *Moringa oleifera*. Pulverized *Moringa oleifera* leaves were sequentially extracted with four solvents. BRIN-BD11 cells were grown in an atmosphere of 5% CO₂ and 95% air in RPMI-1640. Insulin and LDH assays were carried out using Krebs Ringers Bicarbonate buffer. Insulin concentration was measured by radioimmunoassay and LDH release was assessed using a commercially available non-radioactive assay kit (Promega, UK). Aqueous extraction produced the best yield (361 mg/20kg powder). Acute incubation of BRIN-BD11 cells with different extracts of *M. oleifera* produced a concentration-dependent stimulation of insulin-release at concentrations ≥ 10 $\mu\text{g/ml}$. The aqueous extract produced the best insulintropic effect, with a maximum stimulation of 347% ($P < 0.001$) of basal rate at 100 $\mu\text{g/ml}$ and the lowest stimulating concentration of 0.1 $\mu\text{g/ml}$. Dichloromethane and acetone extracts produced similar stimulatory effects (130% of basal rate, 100 $\mu\text{g/ml}$, $P < 0.01$). Insulin-releasing effects of the four extract were not associated with beta cell cytotoxicity. These results showed that extracts of *M. oleifera* possess insulintropic effects and encourage further studies to uncover the physiological mechanism involved.

Keywords: Insulin-releasing, *Moringa oleifera*, BRIN-BD11 cells, cytotoxicity, anti-diabetic

INTRODUCTION

Records abound of the folkloric use of various plant-based preparations in the management of many diseases worldwide (Newman and Craig, 2007). Advances in science and technology exemplified by improved separation and purification techniques have significantly aided scientific investigations of the efficacy of many therapeutic natural products (Conlon *et al*, 2007). In addition, the development of various cell-lines for *in vitro* screening of drug candidates (McClenaghan *et al*, 1996) and various animal models of human diseases (King, 2012) has further encouraged bio-prospecting for novel agents that could cater for unmet needs in the management of many human diseases, including diabetes mellitus. Increasing incidence of diabetes (Wild *et al*, 2004) and several challenges facing its management have motivated the search for novel agents with anti-diabetic effects.

Moringa oleifera is a tropical tree commonly found in Africa, the sub-Himalayan tracts of India, Pakistan, Bangladesh and Afghanistan (Fahey, 2005). In addition to its several other uses, many recent studies have investigated nutritional and medicinal properties of *M. oleifera*, reporting its antibiotic (Caceres *et al*, 1991; Dahot, 1998), antitrypanosomal (Mekonnen *et al*, 1999), hypotensive (Faizi *et al*, 1994), anti-cancer (Costa-Lutofo *et al*, 2005), anti-inflammatory (Ezeamuzie *et al*, 1996), hypocholesterolemic (Mehta *et al*, 2003) and hypoglycemic activities (Kar *et al*, 2003). Several bioactive compounds, including carbamates and thiocarbamates (Francis *et al*, 2004) (Table 1), isothiocyanates and several peptides (Fahey, 2005) have been isolated from the pod, leaves and roots of *M. oleifera*. This study investigated insulin-releasing effects of various extracts of *M. oleifera*, using clonal pancreatic cell-line, BRIN-BD11. We also assessed cytotoxic effects of the extracts, using the release of the cytosolic enzyme, lactate dehydrogenase (LDH) as a bio-marker.

MATERIALS AND METHODS

Plant material preparation

Fresh leaves of *M. oleifera* were collected in August 2013 around Sangere Village in Girei Local Government Area of Adamawa State, Nigeria. After botanical identification, a voucher specimen (No: OO2013-04) was deposited in the herbarium of Chevron Biotechnology Centre, Modibbo Adama University of Technology, Yola. *M. oleifera* leaves were dried at room temperature and pulverized using a pestle and mortar into fine powder. Ground plant material was sequentially extracted with dichloromethane, acetone, ethylacetate and water. Briefly, dichloromethane extract was prepared by soaking 20g of the ground powder in 50ml of the solvent with intermittent shaking for 3 days. The mixture was first filtered with a double-layer of muslin cloth and then Whatman No.1 filter paper. The resultant residue was rinsed twice with dichloromethane and all the filtrates were pooled together. The solvent was removed from the extract at 40°C in an oven. After complete drying, the yield of the extract was ascertained as presented in Table 2. The residue was subjected further extraction sequentially with ethylacetate, acetone and water following the same extraction procedure described for dichloromethane.

***In vitro* insulin-releasing studies**

In vitro insulin-releasing actions of the various extracts of *M. oleifera* were assessed using BRIN-BD11 rat clonal β -cells seeded into 24-well plates (10^5 cells per well). Cells were grown at 37°C in an atmosphere of 5% CO₂ and 95% air in RPMI-1640 supplemented with 10% (v/v) foetal calf serum, antibiotics (100 U/ml penicillin, 0.1mg/ml streptomycin) and 11.1mM glucose as previously described (Abdel-Wahab et al, 2008; Mechkarska et al, 2010; Ojo et al, 2011). Prior to acute tests, cells were pre-incubated for 40 min at 37°C in Krebs Ringers Bicarbonate (KRB) buffer (pH 7.4) supplemented with 0.5% (w/v) BSA and 1.1mM glucose. Test incubations were performed for 20 min at 37°C in the presence of 5.6mM

glucose and graded doses of different *M. oleifera* extracts (0 - 100µg/ml). After incubation, aliquots of cell supernatants were removed for insulin measurement by radioimmunoassay.

Measurement of insulin concentration

Measurement of insulin concentration was carried out as described by Flatt and Bailey (1981) using sodium phosphate (40 mM) supplemented with thiomerosal (0.02% w/v) and BSA (1% w/v). Insulin standard curve was prepared using crystalline rat insulin over a concentration range of 0.039 to 20.0 ng/ml. Insulin antibody (100µl, 1:40,000 dilution) and ¹²⁵I-labelled tracer (100µl, 10,000 cpm per tube) were added in triplicate to insulin standard and unknown samples and incubated for 48 hours at 4°C. Following the addition of a suspension of 5% (v/v) dextran coated charcoal (5% w/v), the mixture was further incubated for 20 min, centrifuged (2500 rpm, 4°C) and decanted for the measurement of free ¹²⁵I-insulin (now bound to charcoal) using a gamma counter. Insulin concentration were interpolated from the standard curve plotted using a spline curve-fitting algorithm using known concentrations of rats insulin.

Assessment of cytotoxicity

To determine cytotoxicity of plant extracts, BRIN-BD11 cells were seeded into 24-multiwell plates as previously described (Ojo et al, 2011). Following overnight culture in RPMI 1640, cells were pre-incubated in KRB buffer containing 1.1 mM glucose (1.0 ml) for 40 min at 37 °C. This was followed by a 20-min incubation with KRB buffer containing 5.6mM glucose in the presence or absence of the various extracts of *M. oleifera* or DMSO (40%). Concentrations of lactate dehydrogenase (LDH) in the cell assay supernatants were measured using a CytoTox96 nonradioactive cytotoxicity assay kit (Promega, Madison, WI, USA) according to the manufacturer's protocol.

Statistical Analysis

Results are expressed as mean \pm S.E.M. and values were compared using two-way ANOVA followed by Newman-Keuls post-hoc test. Groups of data were considered to be significantly different if $P < 0.05$.

RESULT

Extraction yield

Figure 1 presents yield obtained from the sequential extraction of *M. oleifera* leaf powder with the different solvents. Extraction with ethylacetate produced the lowest yield while the highest yield was obtained for aqueous extraction.

Insulin- releasing effects of *M. oleifera* extracts

The basal rate of release of insulin from BRIN-BD11 cells in the presence of 5.6 mM glucose alone was 0.91 ± 0.04 ng/ 10^6 cells/20 min. Addition of alanine (10mM) to the assay buffer increased this rate to 3.14 ± 1.14 ng/ 10^6 cells/20 min ($P < 0.001$; $n = 8$). Effects of the various extracts of *Moringa oleifera* on insulin-release from BRIN-BD11 cells at 5.6mM glucose, shown in Fig. 1, showed that all the extracts produced a significant ($P < 0.05$) and concentration-dependent stimulations of insulin secretion at concentrations $\geq 10\mu\text{g/ml}$. The aqueous extract exhibited the most potent effects, producing a significant stimulation of insulin release (154% of basal rate, $P < 0.05$) from BRIN-BD11 cells at a concentration of $0.1\mu\text{g/ml}$, with a maximum response (347% of basal rate, $P < 0.001$) at a concentration of $100\mu\text{g/ml}$. Maximum insulin-releasing response (130% of basal rate, $100\mu\text{g/ml}$, $P < 0.01$) to acetone and dichloromethane extracts was moderate while a poor stimulatory effect was observed for incubation with ethylacetate extract (120%, $100\mu\text{g/ml}$, $P < 0.05$).

Cytotoxic effects of *M. oleifera* extracts

The release of the cytosolic enzyme, lactate dehydrogenase (LDH), due to damaged plasma membrane was used as a marker of cytotoxicity in this study. Significant ($P < 0.001$) release of the enzyme was observed in incubations involving DMSO (40% v/v). However, incubation with the different extracts of *M. oleifera* produced no significant release of LDH.

DISCUSSION

Pharmacological effects of several phytochemicals have been reported and studies providing scientific explanations for the ethnopharmacological use of many plants are increasing. Activities such as prevention of hyperglycemia (Kar et al, 2003), inhibition of oxidative stress caused by streptozotocin in rats (Yassa and Tokamy, 2014) and prevention of the formation of advanced glycated end-products (Sangkitikomol et al, 2014) have been previously reported for *M. oleifera* extracts. An earlier study has also reported that N-benzyl thiocarbamates, N-benzyl carbamates, benzyl nitriles, and benzyl ester extracted from *M. oleifera* pod stimulated insulin secretion from the insulin secreting cell-line, INS-1 (Francis et al, 2004). Consistent with these studies, our results indicated significant stimulation of insulin-release by the aqueous and acetone extracts of *M. oleifera* leaves from BRIN-BD11 cells. BRIN-BD11 cell-line was generated by electrofusion of RINm5F cells with New England Deaconess Hospital rat pancreatic islet cells (McClenaghan et al, 1996) and has been used extensively to characterize insulinotropic effects of several agents, including crude plant extracts (Hannan et al, 2007), amphibian skin peptides (Ojo et al, 2011) and synthetic analogues of various endogenous peptides such as GIP and GLP-1 (Irwin et al, 2010).

Compared to other extracts, the superior insulinotropic actions of the aqueous extract coupled with significantly higher amount of yield generated following aqueous extraction suggests that the active ingredient in *M. oleifera* leaves might be water soluble. While the aqueous

extracts of *M. oleifera* has been reported to down-regulate the expression of some pro-inflammatory genes and increase cytotoxic effect of chemotherapy in pancreatic cancer cells (Berkovich et al, 2013), no cytotoxicity was observed following the incubation of the aqueous, as well as other, extracts of *M. oleifera* with the clonal pancreatic beta cell, BRIN-BD11. Significant release of LDH in the presence of DMSO (40% v/v) observed in this study is consistent with previous reports of its cytotoxic actions at concentrations above 20% (v/v) (Violante et al, 2002). In addition, this result indicates that the integrity of the plasma membrane had been preserved and the increased insulin secretion observed in the presence of *M. oleifera* extracts reported is not a consequence of damaged plasma membrane, leading to uncontrolled release of cytosolic enzymes.

CONCLUSION

The insulin-releasing effects reported for extracts of *M. oleifera* leaves in this study partly provide preliminary explanation for the antidiabetic effects previously reported for the plant. However, further studies to delineate underlying mechanisms of its insulinotropic actions are needed.

CONFLICT OF INTEREST

Authors declare no conflict of interests

ETHICAL APPROVAL

Not applicable

REFERENCES

- Abdel-Wahab YHA, Power GJ, Flatt PR, Woodhams DC, Rollins-Smith LA and Conlon JM (2008). A peptide of the phylloseptin family from the skin of the frog *Hylomantis lemur* (*Phyllomedusinae*) with potent *in vitro* and *in vivo* insulin-releasing activity. *Peptides* 29:2136-2143
- Berkovich L, Earon G, Ron I, Rimmon A, Vexler A and Lev-Ari S (2013). *Moringa Oleifera* aqueous leaf extract down-regulates nuclear factor-kappaB and increases cytotoxic effect of chemotherapy in pancreatic cancer cells. *BMC Complem Altern Med*. 13:212-219
- Caceres A, Cabrera O, Morales O, Mollinedo P and Mendia P (1991). Pharmacological properties of *Moringa oleifera*. 1: Preliminary screening for antimicrobial activity. *J. Ethnopharmacol* 33: 213-216.
- Conlon JM, Al-Ghaferi N, Abraham B and Leprince J (2007). Strategies for development of naturally occurring antimicrobial peptides into therapeutically valuable anti-infective agents. *Methods* 42:349-357
- Costa-Lotufo LV, Khan MTH, Ather A, Wilke DV, Jimenez PC, Pessoa C, de Moraes MEA and de Moraes MO (2005). Studies of the anticancer potential of plants used in Bangladeshi folk medicine. *J. Ethnopharmacol* 99: 21-30.
- Dahot MU (1998). Antimicrobial activity of small protein of *Moringa oleifera* leaves. *J. Islamic Acad. Sci*. 11(1): 6
- Ezeamuzie IC, Ambakederemo AW, Shode FO and Ekweblem SC (1996). Antiinflammatory effects of *Moringa oleifera* root extract. *Pharm. Biol* 34(3): 207-212
- Fahey JW (2005). *Moringa oleifera*: A Review of the Medical Evidence for Its Nutritional, Therapeutic, and Prophylactic Properties -Part1. *Tree of Life J*. 1:5
- Faizi S, Siddiqui BS, Saleem R, Siddiqui S, Aftab K and Gilani A (1994). Novel hypotensive agents, niazimin A, niazimin B, niazicin A and niazicin B from *Moringa*

- oleifera*: Isolation of first naturally occurring carbamates. *J. Chem. Soc., Perkin Trans* 1: 3035-3040
- Flatt PR and Bailey CJ (1981). Abnormal plasma glucose and insulin responses in heterozygous lean (ob/+) mice. *Diabetologia* 20:573–577
- Francis JA, Jayaprakasam B, Olson LK and Nair MG (2004). Insulin Secretagogues from *Moringa oleifera* with Cyclooxygenase Enzyme and Lipid Peroxidation Inhibitory Activities. *HCA* 87: 317–326.
- Hannan JMA, Ali L, Marenah L, Rokeya B, Flatt PR and Abdel-Wahab YHA (2007). Insulin secretory actions of extracts of *Asparagus racemosus* root in perfused pancreas, isolated islets and clonal pancreatic beta-cells. *J. Endocrinol* 192 (1):159-168.
- Irwin N, Flatt PR, Patterson S and Green BD (2010). Insulin-releasing and metabolic effects of small molecule GLP-1 receptor agonist 6,7-dichloro-2-methylsulfonyl-3-N-tert-butylaminoquinoxaline. *Eur. J. Pharmacol* 628 (1-3):268-273.
- Kar A, Choundhary B and Bandyopadhyay N (2003). Comparative evaluation of hypoglycaemic activity of some Indian medicinal plants in alloxan diabetic rats. *J. Ethnopharmacol* 84(1): 105-108
- King AJ (2012). The use of animal models in diabetes research. *Br J Pharmacol.* 166 (3):877-894
- McClenaghan NH, Barnett CR, Ah-Sing E, Abdel-Wahab YH, O'Harte FP, Yoon TW, Swanson-Flatt SK and Flatt PR (1996). Characterization of a novel glucose-responsive insulin-secreting cell line, BRIN-BD11, produced by electrofusion. *Diabetes* 45(8):1132-1140
- Mechkarska ME, Ahmed L, Coquet, Leprince J, Jouenne T, Vaudry H, King JD and Conlon JM (2010). Antimicrobial peptides with therapeutic potential from skin secretions of the Marsabit clawed frog *Xenopus borealis* (Pipidae), *Comp. Biochem. Physiol. C* 152:467-72

- Mehta LK, Balaraman R, Amin AH, Bafna PA and Gulati OD (2003). Effect of fruits of *Moringa oleifera* on the lipid profile of normal and hypocholesterolaemic rabbits. *J. Ethnopharmacol* 86: 191-195
- Mekonnen Y, Yardley V, Rock P and Croft S (1999). *In vitro* antitrypanosomal activity of *Moringa stenopetala* leaves and roots. *Phytother Res* 13: 538-539
- Newman DJ and Craig GM (2007). Natural products as sources of new drugs over the last 25 years. *J Nat Prod.* 70:461-477.
- Ojo OO, Abdel-Wahab YHA, Flatt P, Mechkarska M and Conlon JM (2011). Tigerinin-1R: a potent, non-toxic insulin-releasing peptide isolated from the skin of the Asian frog, *Hoplobatrachus rugulosus*. *Diabetes Obes Metab* 13(12): 1114 – 1122
- Sangkitikomol W, Rocejanasaroj A and Tencomnao T (2014). Effect of *Moringa oleifera* on advanced glycation end-product formation and lipid metabolism gene expression in HepG2 cells. *Genet Mol Res.* 13(1):723-735
- Violante GDA, Zerrouk N, Richard I, Provot G, Chaumeil JC and Arnaud P (2002). Evaluation of the Cytotoxicity Effect of Dimethyl Sulfoxide (DMSO) on Caco2/TC7 Colon Tumor Cell Cultures. *Biol. Pharm. Bull.* 25(12) 1600—1603
- Wild S, Roglic G, Green A, Sicree R and King H (2004). Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. *Diabetes Care* 27: 1047–1053
- Yassa HD and Tohamy AF (2014). Extract of *Moringa oleifera* leaves ameliorates streptozotocin-induced Diabetes mellitus in adult rats. *Acta Histochem.* doi: 10.1016/j.acthis.2014.02.002

Legend to Figures

Fig. 1: *M. oleifera* extraction with different solvents. DCM = Dichloromethane, ETH = Ethylacetate, ACE = Acetone and AQU = Aqueous

Fig. 2: Effects of various extracts of *M. oleifera* on the release of insulin from BRIN-BD11 clonal β -cells in the presence of 5.6 mM glucose. (***) $P < 0.001$, (**) $P < 0.01$, (*) $P < 0.05$ compared to glucose; $n = 8$).

Fig. 3: Effects of various extracts of *M. oleifera* on lactose dehydrogenase from BRIN-BD11 clonal β -cells in the presence of 5.6 mM glucose.

Fig. 1

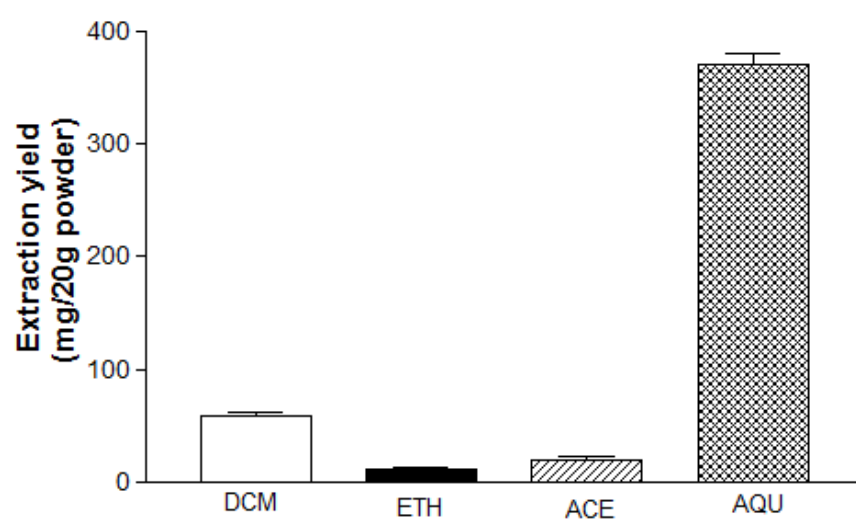


Fig. 2

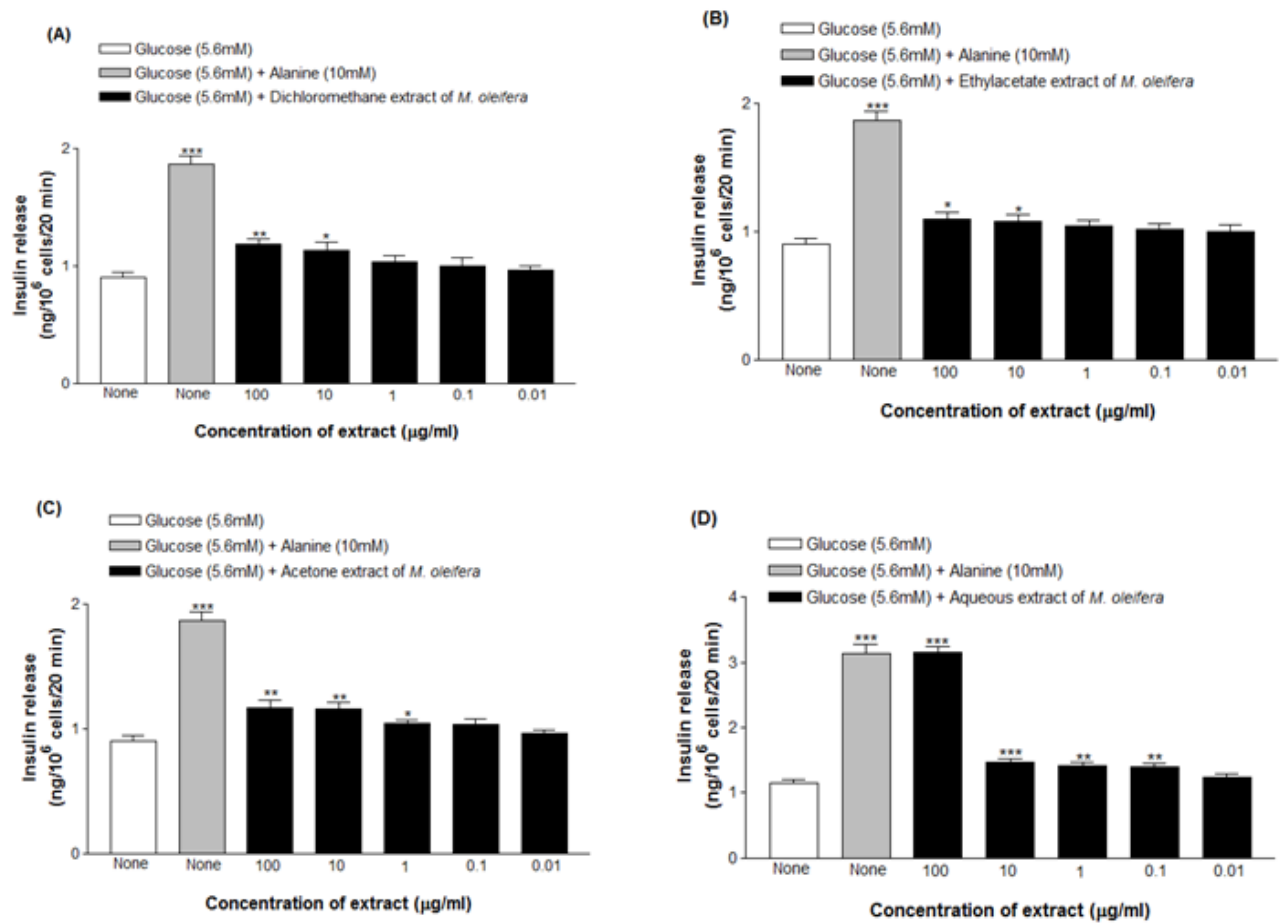


Fig. 3

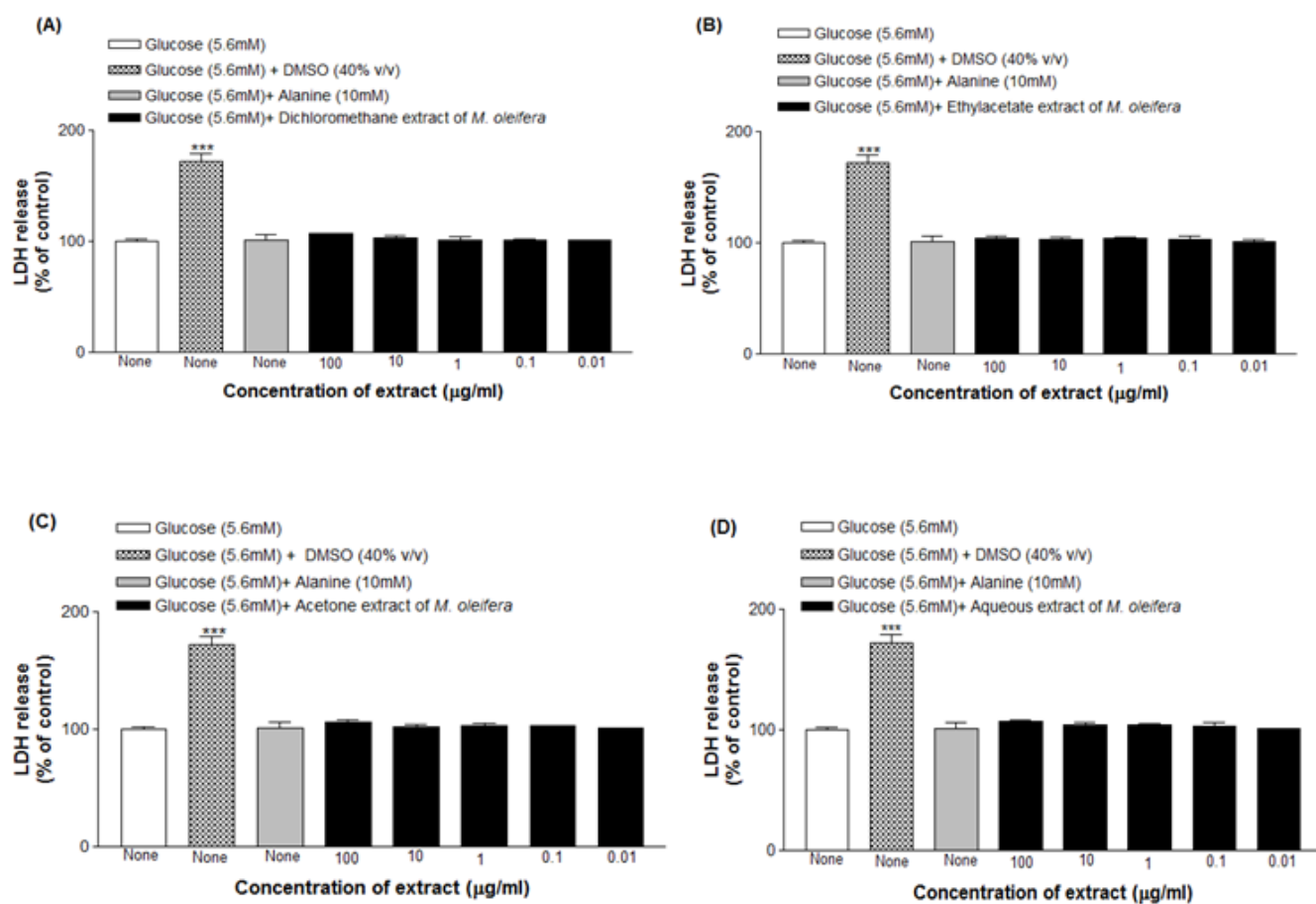


Table 1: Selected bioactive compounds isolated from *Moringa oleifera*

S/No	Name
1	Methyl 2-[4-(α -L-rhamnopyranosyl)phenyl]acetate
2	N-[4-(β -L-rhamnopyranosyl)benzyl]-1-O- α -D-glucopyranosylthiocarboxamide
3	1-O-phenyl- α -D-rhamnopyranoside
4	4-[(β -D-glucopyranosyl)-(1 \rightarrow 3)-(α -L-rhamnopyranosyl)]phenylacetonitrile
5	4-(α -L-rhamnopyranosyl)phenylacetonitrile
6	Methyl N-{4-[(4'-O-acetyl- α -L-rhamnopyranosyl)benzyl]}thiocarbamate
7	Methyl N-{4-[(α -L-rhamnopyranosyl)benzyl]}carbamate
8	Methyl N-{4-[(4'-Oacetyl- α -L-rhamnopyranosyl)benzyl]}carbamate

Francis et al, 2004; Fahey, 2005